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(54) Title: REUSABLE LOW FLUORESCENT PLASTIC BIOCHIP

(57) Abstract

The invention relates to reusable biochips for the detection of target analytes. The biochips comprise an array of ligands attached to a non–fluorescent acrylic that finds use in fluorescence detection and other methodologies.

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REUSABLE LOW FLUORESCENT PLASTIC BIOCHIP

FIELD OF THE INVENTION

The invention relates to novel biochips for the detection of target analytes. The biochips comprise an array of ligands attached to a non-fluorescent acrylic that finds use in fluorescence detection and other methodologies.

BACKGROUND OF THE INVENTION

There are a number of assays employing biochips for the detection of the presence or analysis of specific substances or target analytes. Many of these rely on specific ligand/target analyte interactions. That is, pairs of substances (i.e. the binding pairs or ligand/target analytes) are known to bind to each other, forming complexes, while binding little or not at all to other substances. This has been the focus of a number of techniques that utilize these binding pairs for detection. This generally is done by labeling one component of the complex in some way, so as to make the entire complex detectable, using, for example, radioisotopes, fluorescent and other optically active molecules, enzymes, etc.

One approach to detect ligand/target analyte interactions, utilizes an array of ligands attached to a solid support. Supports for ligand arrays have included porous membranes, such as, nitrocellulose or nylon. Detection methods using porous membranes are limited, owing to autofluorescence and scattering, to radioactive, chemiluminescent, and colorimetric techniques (Ross *et al.* 1992. In *Techniques for the analysis of complex genomes* (ed. Rakesh Anand), pp.137-153. Academic Press, London, UK.) More recently, ligand arrays have been produced on glass surfaces which can be mass produced, certaom of which are not autofluorescent and, therefore, are compatible with fluorescent detection methods. Fluorescent detection is the method of choice when using microarrays because, unlike most

radioactive and chemiluminescent signals, fluorescent signals do not disperse and therefore allow for very dense ligand spacing in the array. Furthermore, glass surfaces when appropriately treated with silane or polylysine exhibit reduced nonspecific binding of target analytes, resulting in lower background than is typically encountered with porous membranes (Shalon *et al.* 1996. 6:639-645). However, silane or polylysine coated slides will not readily withstand harsh conditions necessary to reduce autofluorescence and/or re-use due to the lability of the silane or polylysine coatings to harsh conditions, such as, elevated temperature and/or non-neutral pH, which are required for stripping the arrays of their target analytes after the hybridization and analysis steps.

Accordingly, it is an object of the invention to provide non-autofluorescent solid support that is an alternative to glass that is a suitable for the construction of biochips that can be employed in high-sensitivity, fluorescence detection and other methodologies and that also are reusable.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the invention provides a biochip comprising a biological binding ligand immobilized to a non-fluorescent plastic support.

In a further aspect the invention provides a method of making a biochip comprising a biological binding ligand immobilized to a non-fluorescent plastic support.

In an additional aspect, the invention provides a method of detecting a target analyte in a sample comprising the steps of contacting a sample with a biochip of the invention under conditions that permit binding of the target analyte to at least one biological binding ligand attached to the biochip and detecting the presence of the target analyte.

In another aspect, the invention provides a method of stripping the bound target analyte from the biological binding ligand of the biochip and reuse of the biochip and binding ligand for further detection of a target analyte.

In yet another aspect, the invention provdes a kit comprising a biochip and at least one reagent.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides novel biochips comprising a substantially non-fluorescent plastic support containing an immobilized array of biological binding ligands. The low background fluorescence of this plastic support, in contrast to other plastics, permits the use of the biochip in fluorescence-based methodologies. The novel biochip permits rigorous washing in order to reduce autofluorescence, thereby enhancing the signal to noise ratio.

Accordingly, the present invention provides biochips comprising a plurality of biological binding ligands immobilized onto a substantially non-fluorescent plastic support.

By "substantially nonfluorescent" or "non-fluorescent" herein generally is meant from a low grade or dull fluorescence to completely non-fluorescent, preferably the fluorescence is as low as glass. When used in a fluorescence detection method, the fluorescent light, if emitted from the support, does not interfere or compete with or mask the detection of the fluorescent signal of a ligand/target analyte binding pair assay complex. Thus, for example, either the solid support emits very little light at the wavelength of the fluorescent label used in the assay, or, alternatively, the wavelength at which the solid support does fluorescence is sufficiently different from the emission wavelength of the label so as not to interfere with the detection of the label. Accordingly, solid support/label combinations are preferred. In a preferred embodiment, the emission due to the solid support is only a small percentage of the label signal, i.e. preferably less than about 20% of the signal, with less than about 15% being preferred, and less than about 10 being especially preferred. This can be determined by

taking a reading in an area different from that occupied by the labelled target analyte to give the background fluorescence. In the most prefered embodiment, the solid support is non-fluorescent, i.e. transparent.

Without being bound by theory, it appears that most plastics comprise plasticizers, UV protectants, and other additives that can cause background fluorescence. Accordingly, preferred embodiments utilize plastics that do not comprise these components.

The substantially non-fluorescent solid support is preferably a transparent plastic, such as acrylic or polypentene, and is generally smooth, can have any shape in cross-section and can be rigid or flexible. Preferably, the solid-support as described herein are depicted as a flat surface, which is only one of the possible conformations of the solid support and is for schematic purposes only. The solid support may be a disc, square, sphere, circle, foam of closed-cells, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions. The conformation of the solid support will vary with the detection method used. For example, a flat planar solid-support may be preferred for optical detection methods, or when arrays of biological binding ligands are made, thus requiring addressable locations for both synthesis and detection. Alternatively, the solid support may be in the form of a tube, with the biological binding ligands bound to the inner surface. This allows a maximum of surface area containing the biological binding ligands to be exposed to a small volume of sample. In alternative embodiments, the solid support is rigid or soft. A soft support is easily manipulated to assume a variety of configurations. Preferably, the solid support is easily manipulated, cut or reshaped before or after arrayed. In addition, plastic has an advantage over glass in that it will not shatter. In additional advantage, plasic supports can be re-positioned under or relative to a fixed detector.

The solid support also does not react adversely to chemicals and conditions used in the immobilization of the ligand array such that its physical, chemical properties, and opitcal properties are adversely altered. The solid support also does not react adversely to chemicals

and conditions used in the formation, detection, or removal of ligand/target analyte complexes. Therefore, the solid support can be manipulated as described below for the immobilization of the binding ligands for the construction of biochips that find use in a number of assays and techniques as described below.

Accordingly, the solid support is resistant to temperatures preferably of from about 0°C to about 100°C, more preferably from about 10°C to about 90°C, and even more preferably from about 20°C to about 80°C.

The solid support also is resistant to pH preferably from about 1 to about 14, and more preferably of about 5 to about 14. In addition, as is more fully outlined below, the resistance of the chips to high concentrations of base, i.e. 1 M NaOH, confers a number of benefits, including the ability to completely remove all non-covalently bound biomolecules.

Accordingly, the solid support is resistant to the chemicals and reagents used in either the generation of the biochip or the assay itself.

By "resistant" and grammatical equivalents herein is meant that the optical, physical, and chemical properties of the solid support are not substantially altered in the construction, storage, or use of the biochip in such a way as to interfere or inhibit the immobilization of the ligand array or the detection and analysis of the target analyte.

In a preferred embodiment, the solid support is made of commercial grade close tolerance, UVT cast acrylic, available from Glasflex, (Glasflex, 4 Stirling Road, Stirling, New Jersey 07980). Cast acrylic is preferable, as extruded acrylic presumably comprises plasticizers and other components that may cause background fluorescence.

In general, a potential solid support candidate is tested by scanning an area of the material at the wavelength of interest (i.e. the wavelength at which the biomolecule label fluoresces) and

comparing it to a scan of the same area of a glass substrate, with those materials showing similar or less fluorescence being "substantially non-fluorescent" as defined herein.

Preferably, the solid support is not made of substances having a substantial background fluoresence, such as, polymethylpentene (Westlake: TPX), polycarbonate (Westlake: Zelux), polyetherimide (Westlake: Ultem), polyphenylene oxide (Westlake: Noryl) , polyvinylidene fluoride (Westlake: Kynar), acetal (Westlake: Pomalux), Hytrel (Westlake), Ultraform (Westlake), lucite (ICI), clear acrylic (AIN plastic) , black acrylic (Allen extruders), clear acrylite (AIN), polypropylene (Sigma slides), extruded clear acrylic, black polystyrene, polycarbonate ZX (Cyro Industries), cell cast acrylite GP (Cyro Industries), impact modified acrylic colorless HP (Cyro Industries), continuously manufactured acrylic (Cyro Industries: Acrylite).

By "biochip" or grammatical equivalents herein is meant a composition used to detect at least one target analyte. By "target analyte" or "analyte" or grammatical equivalents herein is meant any molecule, compound, particle, cell or substance to be detected. As outlined below, target analytes preferably bind to at least one biological binding ligand. As will be appreciated by those in the art, a large number of analytes may be detected using the present methods; basically, any target analyte for which a biological binding ligand, described herein, may be made may be detected using the methods of the invention.

The biochip comprises a plurality of biological binding ligands. By "plurality" and grammatical equivalents herein is meant at least two biological binding ligands. By "biological binding ligand" or grammatical equivalents herein is meant a compound that is used to probe for the presence of the target analyte, and that will bind to the analyte. As will be appreciated by those in the art, the present invention allows the generation of arrays. By "array" and grammatical equivalents herein is meant a plurality of biological binding ligands in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different biological binding ligands to many millions can be made, with very large arrays being possible. Generally, the array will comprise from

two to as many as about 100,000 (all densities are per square cm) or more, depending on biological binding ligand, the size of the solid support, as well as the end use of the array, thus very high density, high density, moderate density, low density and very low density arrays may be made. Preferred ranges for high density arrays are from about 10K to about 125K. Moderate density arrays range from about 1K to about 10K being preferred Low density arrays are generally less than 1K, with from about 0.1K to about 1K being preferred. Very low density arrays are less than 0.1K. In some embodiments, the biological binding ligands of the invention may not be in array format; that is, for some embodiments, biochips comprising a single ligand may be made as well. In addition, in some arrays, multiple solid supports may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller solid supports.

As will be appreciated by those in the art, the composition of the biological binding ligand will depend on the composition of the target analyte. Biological binding ligands for a wide variety of analytes are known or can be readily found using known techniques. For example, when the analyte is a protein, the biological binding ligands include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)), small molecules, peptide "aptamers", or peptidomimmetic structures. When the analyte is a metal ion, the binding ligand generally comprises traditional metal ion ligands or chelators. Preferred biological binding ligand proteins include peptides. For example, when the analyte is an enzyme, suitable binding ligands include substrates and inhibitors. Antigen-antibody pairs, receptor-ligands, and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. The binding ligand may be nucleic acid, when nucleic acid binding proteins are the targets; alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptamers" can be developed for binding to virtually any target analyte. Similarly, there is a wide body of literature relating to the development of biological binding ligands based on combinatorial chemistry methods. In a preferred embodiment, the biological binding ligand is a nucleic acid.

Suitable analytes include organic and inorganic molecules, including biomolecules. In a preferred embodiment, the analyte may be an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc.; nucleic acids; proteins (including enzymes, antibodies, antigens, growth factors, cytokines, etc); therapeutic and abused drugs; cells; viruses; protozoa; and other infectious agents.

In a preferred embodiment, the target analyte is a nucleic acid, i.e. a nucleic acid target sequence. The term "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be chemically synthesized nucleic acid, such as, an oligonucleotide or a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art. The target sequence may also be comprised of different target domains, for example, a first and second target domain which may be adjacent or separated. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the entire target sequence. For example, assuming a 5'-3' orientation of the complementary target sequence, the first target domain may be located either 5' to the second domain, or 3' to the second domain.

In a preferred embodiment, the target analyte is a protein. By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline and norleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. The side chains may be in either the (R) or the (S) or L configuration. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations.

Suitable protein target analytes include, but are not limited to, (1) immunoglobulins, particularly IgEs, IgGs and IgMs, and particularly therapeutically or diagnostically relevant antibodies, including but not limited to, for example, antibodies to human albumin, apolipoproteins (including apolipoprotein E), human chorionic gonadotropin, cortisol, α-fetoprotein, thyroxin, thyroid stimulating hormone (TSH), antithrombin, antibodies to pharmaceuticals (including antiepileptic drugs (phenytoin, primidone, carbariezepin, ethosuximide, valproic acid, and phenobarbitol), cardioactive drugs (digoxin, lidocaine, procainamide, and disopyramide), bronchodilators (theophylline), antibiotics (chloramphenicol, sulfonamides), antidepressants, immunosuppresants, abused drugs (amphetamine, methamphetamine, cannabinoids, cocaine and opiates) and antibodies to any number of viruses (including orthomyxoviruses, (e.g. influenza virus), paramyxoviruses (e.g respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (e.g. rubella virus), flaviviruses (e.g. yellow fever virus), parvoviruses, poxviruses (e.g. variola virus, vaccinia virus), enteroviruses (e.g. poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (e.g. Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus), rotaviruses, Norwalk viruses, bunyavirus (e.g., hantaan virus), arenavirus, rhabdovirus (e.g. rabies virus), retroviruses (including HIV, HTLV-I and -II), papovaviruses (e.g.

papillomavirus), polyomaviruses, and picornaviruses, filoviruses (e.g. Ebola virus), and the like); bacteria (including a wide variety of pathogenic and non-pathogenic prokaryotes of interest including Bacillus; Vibrio, e.g. *V. cholerae;* Escherichia, e.g. Enterotoxigenic *E. coli*, Shigella, e.g. *S. dysenteriae;* Salmonella, e.g. *S. typhi;* Mycobacterium e.g. *M. tuberculosis, M. leprae*; Clostridium, e.g. *C. botulinum, C. tetani, C. difficile, C.perfringens*; Cornyebacterium, e.g. *C. diphtheriae*; Streptococcus, *S. pyogenes, S. pneumoniae;* Staphylococcus, *e.g. S. aureus*; Haemophilus, e.g. *H. influenzae*; Neisseria, e.g. *N. meningitidis, N. gonorrhoeae*; Yersinia, e.g. *Y. pestis*, Pseudomonas, e.g. *P. aeruginosa, P. putida*; Chlamydia, e.g. *C. trachomatis*; Bordetella, e.g. *B. pertussis;* Treponema, e.g. *T. palladium;* Rickettsia, e.g. *R. prowazekii, R. ricketsii* and the like); and protozoa (including Giardia e.g. *G. lamblia*, Entamoeba, *E. histolytica;* Plasmodia e.g. *P. vivax, P. falciparum*; Leishmania, e.g., *L. donovanii, L. braziliensis*; Trypanosoma, e.g. *T. cruzi* and the like); (2) enzymes (and other proteins), including but not limited to, enzymes used as indicators of or treatment for heart disease, including creatine kinase, lactate dehydrogenase, aspartate amino transferase, troponin T, myoglobin, fibrinogen, cholesterol, triglycerides, thrombin, tissue plasminogen activator (tPA); pancreatic disease indicators including amylase, lipase, chymotrypsin and trypsin; liver function enzymes, proteins, disease indicators including cholinesterase, bilirubin, and alkaline phosphatase; aldolase, prostatic acid phosphatase, terminal deoxynucleotidyl transferase, and bacterial and viral enzymes such as HIV protease; (3) hormones and cytokines (many of which serve as ligands for cellular receptors) such as erythropoietin (EPO), thrombopoietin (TPO), the interleukins (including IL-1 through IL-17), insulin, insulin-like growth factors (including IGF-1 and -2), epidermal growth factor (EGF), transforming growth factors (including TGF-α and TGF-β), human growth hormone, transferrin, epidermal growth factor (EGF), low density lipoprotein, high density lipoprotein, leptin, VEGF, PDGF, ciliary neurotrophic factor, prolactin, adrenocorticotropic hormone (ACTH), calcitonin, human chorionic gonadotropin, cortisol, estradiol, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), leutinzing hormone (LH), progesterone, testosterone; and (4) other proteins (including α-fetoprotein, carcinoembryonic antigen CEA.

In a preferred embodiment, the target analyte is a carbohydrate. Suitable carbohydrate analytes include but are not limited to, markers for breast cancer (CA15-3, CA 549, CA 27.29), mucin-like carcinoma associated antigen (MCA), ovarian cancer (CA125), pancreatic cancer (DE-PAN-2), and colorectal and pancreatic cancer (CA 19, CA 50, CA242). Other suitable carbohydrate analytes include, for example, carbohydrate moieties which bind to lectins.

In addition, any of the target analytes for which antibodies may be detected may be detected directly as well; that is, detection of virus, bacterial cells, protozoa and other microbes, therapeutic and abused drugs, etc., may be done directly.

In a preferred embodiment, the target analyte is a metal. Suitable metal analytes include metal ions, particularly heavy and/or toxic metals, including but not limited to, aluminum, arsenic, cadmium, selenium, cobalt, copper, chromium, lead, silver and nickel.

In a preferred embodiment, the target analyte is a nucleic acid target sequence and the biological binding ligand is a nucleic acid probe. By "nucleic acid" and grammatical equivalents herein is meant two or more nucleotides joined together. The nucleic acid analyte can be DNA, RNA, or cDNA, and can be naturally occurring or synthetic as well as analogs thereof. The nucleic acid can have any combination of natural and synthetic bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine, halogenated bases, etc. Also included in the definition of nucleic acid are nucleic acid analogs that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical

Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference.

The nucleic acid probes of the present invention are designed to be complementary to a target sequence (either the target sequence of the sample or to other probe sequences, as is described below, for example in sandwich assays), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, as outlined below.

A nucleic acid probe is generally single or double stranded but can be partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. A nucleic acid probe of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined above, nucleic acid analogs are included that may have alternate backbones or synthetic bases. As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and nucleic acid analogs can be made.

When nucleic acids are used as the biological binding ligands for binding of a target analyte, the nucleic acid probes range from about 6 to about 150 basepairs long, with from about 10 to about 100 base pairs being preferred, and from about 12-15 to about 75 bp being particularly preferred and 50 bp being the most preferred. In some embodiments, much longer nucleic acids can be used, up to hundreds of basepairs, for example, partial or full length cDNAs.

A protein biological binding ligand of the present invention is generally a protein, oligopeptide and peptide, derivatives and analogs, including proteins containing non-naturally occurring amino acids and amino acid analogs, and peptidomimetic structures, as described above. As discussed below, when the protein is used as a binding ligand, it may be desirable to utilize protein analogs to retard degradation by sample components or contaminants.

When proteins are used as the biological binding ligands for binding of a target analyte, the proteins range from about 5 to about 200 amino acids long, with from about 10 to about 100 amino acids being preferred. In some embodiments, much longer proteins can be used, up to hundreds of amino acids.

In a preferred embodiment, the binding of the target analyte to the biological binding ligand is specific, and the biological binding ligand is part of a binding pair. By "specifically bind" herein is meant that the ligand binds the analyte, with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. However, as

will be appreciated by those in the art, it will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands. This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, for example in the detection of certain biomolecules, the disassociation constants of the analyte to the biological binding ligand will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-10} M^{-1} being preferred, less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred and 10^{-10} -10^{-11} M^{-1} being most preferred.

Generally, the biochips are made as follows. As will be appreciated by those in the art, the method of immobilizing the ligand arrays of the solid-supports of the present invention may vary. Preferred methods are outlined herein and are known in the art.

The biological binding ligands are immobilized to a substantially non-fluorescent plastic solid support. By "immobilized" and grammatical equivalents herein is meant the association or binding between the biological binding ligand and the solid support is sufficient to be stable under the conditions of target analyte binding, washing, analysis, and removal as outlined below. The binding can be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents herein is meant one or more of either electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the support and the non-covalent binding of biotinylated biological binding ligand to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the biological binding ligand, are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds. Covalent bonds can be formed directly between the biological binding ligand and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the biological binding ligand or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

In general, the biological binding ligands can be attached to the biochip in a wide variety of ways, as will be appreciated by those in the art. In general, the biological binding partner may be either synthesized on the chip, or can be synthesized first and attached later. In either case, the surface of the biochip and the biological binding ligand may be derivatized with chemical functional groups for subsequent attachment of the two. Thus, for example, the biochip is derivatized with a chemical function group including, for example, amino groups, carboxy groups, oxo groups, thiol groups, and N-oxysuccinimide. Using these functional groups, the binding ligands can be attached using functional groups on the binding ligands. For example, proteins and nucleic acids containing amino groups can be attached to modified surfaces, as described below or, for example using linkers as are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). In addition, in some cases, linkers may be used as is described below.

In a preferred embodiment, the biological binding ligands are oligonucleotides, i.e. nucleic acid probes. As described below, the nucleic acids can either be synthesized first, with subsequent attachment to the biochip, or can be directly synthesized on the biochip.

In a preferred embodiment, the oligonucleotides are synthesized as is known in the art, and then attached to the surface of the solid support. As will be appreciated by those skilled in the art, either the 5' or 3' terminus may be attached to the solid support, or attachment may be via an internal nucleoside.

Methods of modifying the surface of the solid support include, for example, photochemical coupling, plasma treatment, chemical etchants, and chemical grafting.

In a preferred embodiment, the composition of the surface and the method of attachment is as described in U.S. Patent Nos. 5,427,779; 4,973,493; 4,979,959; 5,002,582; 5,217,492; 5,258,041 and 5,263,992, and references cited therein, all of which are hereby expressly incorporated by reference. Briefly, coupling of oligonucleotides to the reactive surface of the

solid-support can proceed by a number of methods. For example, photochemical coupling can proceed in one of two ways: a) the oligonucleotide is derivatized with a photoreactive group, followed by attachment to the surface; or b) the surface is first treated with a photoreactive group, followed by application of the oligonucleotide. Preferably, the activating agent comprises a N-oxysuccinimide group (NOS) linked to a photoactive group, that upon exposure to light generates a free radical which then inserts into carbon-carbon bonds of the surface, which is put on the surface first. The NOS group is thus exposed followed by attachment of an N-terminal amino-modified oligonucleotide, as is generally described in Amos et al., Surface Modification of Polymers by Photochemical Immobilization, The 17th Annual Meeting of the Society of Biomaterials, May 1991, Scottsdale AZ, hereby expressly incorporated by reference.

In an alternative embodiment, aminated oligonucleotides can be attached to aldehyde surfaces (reductive chemistry) or can be attached to carboxy surfaces via carbodiimide mediated condensation. Phosphorothioated oligos can be attached, for example, to bromoacetyl surfaces via a nucleophylic substitution or can be attached to a maleimide surface via the -SH moiety. Phosphorylated oligos can be attached for example to an amine surface by carbodiimide mediate condensation.

These particular methods allow the use of a large excess of an oligonucleotide, preferably under saturating conditions; thus, the density and uniformity of the application is high.

In an additional embodiment, the immobilization to the solid support may be very strong, yet non-covalent. For example, biotinylated oligonucleotides can be made, which bind to surfaces covalently coated with streptavidin, resulting in attachment. In another embodiment, the biological binding ligand is immobilized to the solid support that is coated by an antibody. In an additional embodiment, the biological binding ligand is immobilized to the solid support that is coated by a polyamino acid, for example, polylysine.

Alternatively, the oligonucleotides may be synthesized on the surface, as is known in the art. Preferred methods are outlined herein and are known in the art; see WO 95/25116; WO 95/35505; U.S. Patent Nos. 5,700,637 and 5,445,934; and references cited within, all of which are expressly incorporated by reference.

In addition, photoactivation techniques utilizing photopolymerization compounds and techniques are used. In a preferred embodiment, the nucleic acids can be synthesized in situ, using well known photolithographic techniques, such as those described in U.S. Patent No. 5,445,934 and related applications and publications; these methods of attachment form the basis of the Affimetrix GeneChip™ technology.

The attachment of other types of biological binding ligands, such as proteins, is well known in the art.

Linker moieties may also be used to immobilize the biological binding ligand to the solid support. By "linker moieties" or grammatical equivalents, herein is meant molecules which serve to immobilize the biological binding ligand at a distance from the solid support. Linker moieties have a first and a second end. In one embodiment, branched linker moieties are preferred. The first end of the linker moiety is used to covalently attach the linker moiety to the solid support. The second end is used for attachment to the biological binding ligand; for example, preferred linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups being preferred. Preferred functional groups include but are not limited to esters, amide, amine, aldehyde, bromoacetyl, carboxy, oxo, thiol, N-oxysuccinimide, maleimide, epoxy groups and ethylene glycol and derivatives. Using these functional groups, the binding ligands can be attached to the surface. For example, proteins and nucleic acids containing amino groups can be attached to modified surfaces, as described herein or, for example using linkers as are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference).

In this way, binding ligands including proteins, lectins, nucleic acids, small organic molecules, carbohydrates, etc. can be covalently attached to linker moieties which are attached to the surfaces of the invention.

Once made, the compositions of the invention are useful in a wide variety of applications. In a preferred embodiment, the compositions of the invention find use in the detection of target analytes in test samples. Thus, the compositions of the present invention may be used in a variety of research, clinical, quality control, or field testing settings and other known applications (see for example Chetverin et al., Bio/Technology, Vol. 12, November 1994, pp1034-1099, (1994)).

If required, the target analyte is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, electroporation, etc., with purification and/or amplification such as PCR occuring as needed, as will be appreciated by those in the art.

In a preferred embodiment, the target analyte is labeled with, for example, a fluorescent, a chemiluminescent, a chemical, or a radioactive signal, to provide a means of detecting the target analyte's specific binding to a biological binding ligand. Examples of fluorescent labels include Cy-3 and Cy-5. The label also can be an enzyme, such as, alkaline phosphatase or horseradish peroxidase, which when provided with an appropriate substrate produces a product that can be detected. Alternatively, the label can be a labeled compound or small molecule, such as an enzyme inhibitor, that binds but is not catalyzed or altered by the enzyme. The label also can be a moiety or compound, such as, an epitope tag or biotin which specifically binds to streptavidin. For the example of biotin, the streptavidin is labeled as described above, thereby, providing a detectable signal for the bound target analyte. As known in the art, unbound labeled streptavidin is removed prior to analysis.

In an alternative embodiment, the target analyte binds to a biological binding ligand, unbound contaminants are removed, and the bound target analyte is detected using second ligand of the

target analyte that is labeled, for example, labeled antibody, nucleic acid or other compound that specifically binds the target analyte. In an alternative embodiment, the antibody, nucleic acid or compound labeled with an epitope tag or moiety, such as biotin, as described above and which is detected with, for example, a labeled streptavidin. Additional method of improving target analyte detection or sensitivity by building-up of specific complexes is well-known in the art.

In a preferred embodiment, the biochips are used to detect or quantify the presence of target nucleic acid sequences. These assays can be direct hybridization assays or can comprise "sandwich assays", which include the use of multiple probes, as is generally outlined in U.S. Patent Nos. 5,681,702, 5,597,909, 5,545,730, 5,594,117, 5,591,584, 5,571,670, 5,580,731, 5,571,670, 5,591,584, 5,624,802, 5,635,352, 5,594,118, 5,359,100, 5,124,246, 5,681,697, and 5,175,270 and Nilsen et al., J. Theor. Biol. 187:273 (1997), all of which are incorporated herein by reference. In this embodiment, in general, the target nucleic acid is prepared as outlined above, and then added to the biochip comprising a plurality of nucleic acid probes, under conditions that allow the formation of an assay complex (in the case of nucleic acid, this can also be referred to as a hybridization complex).

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al, hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at

equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

Thus, the assays are generally run under stringency conditions which allows formation of the label probe hybridization complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration pH, organic solvent concentration, etc.

These parameters may also be used to control non-specific binding, as is generally outlined in U.S. Patent No. 5,681,697. Thus it may be desirable to perform certain steps at higher stringency conditions to reduce non-specific binding.

The reactions outlined herein may be accomplished in a variety of ways, as will be appreciated by those in the art. Components of the reaction may be added simultaneously, or sequentially, in any order, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used, depending on the sample preparation methods and purity of the target.

In a preferred embodiment, the biochips are used in genetic diagnosis. For example, biochips can be made using the techniques disclosed herein to detect target sequences such as the gene for nonpolyposis colon cancer, the BRCA1 breast cancer gene, P53 and mutants thereof, which is a gene associated with a variety of cancers, the Apo E4 gene that indicates a greater risk of Alzheimer's disease, allowing for easy presymptomatic screening of patients, mutations in the cystic fibrosis gene, or any of the others well known in the art.

The present invention also finds use as a methodology for the detection of mutations or mismatches in target nucleic acid sequences. For example, recent focus has been on the analysis of the relationship between genetic variation and phenotype by making use of polymorphic DNA markers. Previous work utilized short tandem repeats (STRs) as polymorphic positional markers; however, recent focus is on the use of single nucleotide polymorphisms (SNPs), which occur at an average frequency of more than 1 per kilobase in human genomic DNA. Some SNPs, particularly those in and around coding sequences, are likely to be the direct cause of therapeutically relevant phenotypic variants. There are a number of well known polymorphisms that cause clinically important phenotypes; for example, the apoE2/3/4 variants are associated with different relative risk of Alzheimer's and other diseases (see Cordor et al., Science 261(1993). Multiplex PCR amplification of SNP loci with subsequent hybridization to oligonucleotide arrays has been shown to be an accurate and reliable method of simultaneously genotyping at least hundreds of SNPs; see Wang et al., Science, 280:1077 (1998); see also Schafer et al., Nature Biotechnology 16:33-39 (1998). The compositions of the present invention may easily be substituted for the arrays of the prior art.

In an additional embodiment, microbial detection, such as for viruses and bacteria, is done using the complexes of the invention. In this embodiment, probes are designed to detect target sequences from a variety of bacteria and viruses. For example, current blood-screening techniques rely on the detection of anti-HIV antibodies. The methods disclosed herein allow for direct screening of clinical samples to detect HIV nucleic acid sequences, particularly

highly conserved HIV sequences. In addition, this allows direct monitoring of circulating virus within a patient as an improved method of assessing the efficacy of anti-viral therapies. Similarly, viruses associated with leukemia, HTLV-I and HTLV-II, may be detected in this way. Bacterial infections such as tuberculosis, chlamydia, and other sexually transmitted diseases, may also be detected, for example using ribosomal RNA (rRNA) as the target sequences.

In a preferred embodiment, the biochips of the invention find use in the screening of water and food samples for toxic bacteria and parasites. For example, samples may be treated to lyse the bacteria or parasites to release their nucleic acid (particularly rRNA or DNA), and then probes designed to recognize bacterial strains, including, but not limited to, such pathogenic strains as, *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Leishmania*, enterotoxic strains of *E. coli*, and Legionnaire's disease bacteria. Similarly, bioremediation strategies may be evaluated using the compositions of the invention.

In a further embodiment, the biochips are used for forensic "DNA fingerprinting" to match crime-scene DNA against samples taken from victims and suspects.

In an additional embodiment, the biological binding ligands in an array are used for sequencing by hybridization.

Alternatively, the compositions of the invention are useful to detect successful gene amplification in PCR, thus allowing successful PCR reactions to be an indication of the presence or absence of a target sequence. PCR may be used in this manner in several ways. For example, in one embodiment, the PCR reaction is done as is known in the art, and then added to a biochip of the invention. Preferrably, PCR product is labeled to provide a means to detect the PCR product when bound to a biological binding ligand. The PCR label can be bound to one or more of the oligonucleotide primers or can be incorporated into the PCR product during polymerization. The label also can be detected directly or indirectly. By "label" herein is generally meant a substance that directly emits or produces a detectable

signal, such as, an enzyme, a fluorophore, a chemiluminescent compound, a radioactivity isotope and the like. In another embodiment, the label is a ligand or moiety, such as, biotin, that is specifically recognized and bound by a second substance or compound that emits a detectable signal.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All patents, patent applications, publications, and references cited herein are expressly incorporated by reference in their entirety.

EXAMPLES

Example 1

Biochip Preparation

To prepare biochips, 28 pmoles/µl or 28 µM 5'-aminolink oligonucleotide (TFA Aminolink CE phosphoramidite (PEBiosystems PN 402872; also available from Glen Research)) in Array Solution (50 mM Na_2HPO_4, pH 8.5-9.0 , 10% Na_2SO_4; 1 mM EDTA; 0.0005% SDS) was placed into each well of a 384-well plate. In this Example, 10 µl of oligonucleotide/Array Solution was placed into each well. The lid of the plate was labeled with the oligonucleotide synthesis run in the plate/date descriptor. The bottom of the plate was also labeled with the date and plate number. The plate was mixed on a multitube vortexer and was covered in foil and stored in a cold box (4°C) when arraying on the same day. Alternatively, the plates were stored in a -20°C freezer until use.

The biochips were individually dated and numbered. The array area of the biochip also is marked to indicate the placement of a coverslip used for binding to a target analyte. N-oxysuccinamide (NOS) was added to the biochips at Surmodics (9924 West 74th Street, Eden Prairie, Minnesota 55344-3523) as outlined above prior to oligonucleotide transfer. Transfer and arraying of the oligonucleotide from the multiwell plates to the biochips was performed

using a XYZ stage (Norgren Systems) using pins purchased from Telechem International (San Jose, CA).

The transferred oligonucleotides were coupled to the biochips by incubation at 37°C at high humidity (up to 100% humidity) for 2 hours or by incubation at 25°C at 78% humidity for at least 12 hours. Unbound NOS groups were blocked with ethanolamine (0.5 M Tris, 1 N ethanolamine, pH 9) by: i) vigorously dunking biochips in ethanolamine; ii) incubating biochips at 60°C for one hour in a black slide box; and iii) placing biochips on an orbital shaker for 15 minutes. The biochips were rinsed three times in deionized H_2O; stripped with 1 N NaOH in black slide boxes at 70°C for 1 hour; washed four times in deionized H_2O; and spun dry in racks at 1000 RPM (110Xg). Slides were stored in slide boxes until needed.

CLAIMS

1. A biochip comprising a plurality of biological binding ligands immobilized to a non-fluorescent plastic support.

2. A biochip according to claim 1, wherein said biological binding ligands comprise nucleic acids.

3. A biochip according to claim 2, wherein at least one of said nucleic acids is a nucleic acid analog.

4. A biochip according to claim 1, wherein said biological binding ligand is a protein.

5. A biochip according to claim 1, wherein said plastic support is acrylic.

6. A method of making a biochip comprising immobilizing a plurality of biological binding ligands to a non-fluorescent plastic support.

7. A method according to claim 6, wherein said biological binding ligands comprise nucleic acids.

8. A method according to claim 7, wherein at least one of said nucleic acids is a nucleic acid analog.

9. A method according to claim 6, wherein said biological binding ligands are proteins.

10. A method according to claim 6, wherein said plastic support is acrylic.

11. A method of detecting the presence of a target analyte in a sample comprising:

a) contacting said sample with a biochip according to claim 1 under conditions that permit binding of said target analyte to at least one of said biological binding ligands; and

b) detecting the presence of said target analyte.

12. A method according to claim 11, wherein said target analyte comprises a label and said detecting is done by detecting said label.

13. A method according to claim 12, wherein said label is a fluorescent label.

14. A method according to claim 11, further comprising adding a second ligand that is labeled and binds specifically to said target analyte and said detecting is done by detecting a signal produced by said label.

15. A method according to claim 14, wherein said signal is a fluorescent signal.

16. A method according to claim 11, wherein said target analyte is a nucleic acid.

17. A method according to claim 11, wherein said target analyte is a protein.

18. A method according to claim 11 further comprising:

c) stripping the target analyte from said biological binding ligand.

19. A method according to claim 18 further comprising:

d) repeating steps a) and b).

20. A kit comprising a biochip according to claim 1 and at least one reagent.

INTERNATIONAL SEARCH REPORT

Inter[illegible]nal Application No
PC[illegible] 00/06866

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/545 C12Q1/68 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 30774 A (BECKMAN INSTRUMENTS, INC.) 16 November 1995 (1995-11-16) abstract page 4, line 15 - line 33; claims 1,10 ---	1-20
Y	EP 0 063 810 A (CIBA GEIGY AG) 3 November 1982 (1982-11-03) abstract; claims ---	1-20
Y	EP 0 294 105 A (PALL CORPORATION) 7 December 1988 (1988-12-07) column 1 -column 4; claims ---	1-20
Y	EP 0 882 980 A (COMMISSARIAT A L'ENERGIE ATOMIQUE) 9 December 1998 (1998-12-09) the whole document --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention.

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 July 2000	28/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Griffith, G

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INTERNATIONAL SEARCH REPORT

Inter[redacted]onal Application No
[redacted]T/US 00/06866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE 197 39 119 A (FRIEDRICH-SCHILLER-UNIVERSITÄT JENA) 11 March 1999 (1999-03-11) the whole document	1-20

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]nal Application No
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